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In-vitro Androgenesis in Rice: Advantages, Constraints and Future Prospects

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Abstract: *In vitro* androgenesis is an important component of plant biotechnology when the pollen grains are forced to switch from their normal pollen developmental pathway towards an embryogenic route. Haploid and doubled haploid produced through androgenesis have long been recognized as a valuable tool in plant breeding as it can shorten the breeding cycle, fix agronomic characters in homozygous state and enhance the selection efficiency of useful recessive agronomic traits. Recently, doubled haploids have been largely recognized as an important component of crop improvement through genome mapping, quantitative trait locus analysis, and genetic mutation, and as targets for genetic transformation programs. Thus, this review is focused mainly on various facets of doubled haploid in the chief staple food crop rice and sights its recent applications in plant breeding, genetics and genomics.

Key words: *in vitro* androgenesis; doubled haploid; haploid; rice

Doubled haploids (DHs) are produced when haploid cells undergo spontaneous or induced chromosome duplication. *In vitro* induction of maternal haploids or gynogenesis is an efficient approach towards production of haploid embryos from un-pollinated flower parts such as ovules, placenta attached ovules and whole flower buds (Murovec and Bohanec, 2012). It also provides an alternative source for haploid production in plants with predominant male sterility (Bhat and Murthy, 2007). However, poor regeneration and low efficiency of haploids have been the most serious limiting factors in rice and other cereal crops. Although plants have the natural tendency of generating haploid, *in vitro* anther culture or androgenesis is the most efficient and simplest technique used so far to produce haploids/doubled haploids in several species (Forster et al, 2007). Doubled haploid breeding through anther culture has emerged as an exciting and powerful tool, and a convenient alternative to conventional techniques for crop improvement (Purwoko et al, 2010). Doubled haploids have numerous advantages, such as shorten breeding cycle by immediate fixation of homozygosity, high selection efficiency, widen genetic variability through the production of gametoclonal variants, and earlier expression of recessive genes suitable for breeding (Devaux and Pickering, 2005). Presently, more than 250 plant species and hybrids have been regenerated using anther culture technique, which include major cereals such as rice, wheat, maize, barley and a range of economically important trees, fruit crops and medicinal plants (Maluszynski et al, 2003). There are many reviews explaining the production and application of doubled haploids through androgenesis (Forster et al, 2007; Touraev et al, 2009; Seguí-Simarro, 2010; Germana, 2011). However, there are only a limited number of reports highlighting the development of rice doubled haploids and their exploitation in molecular breeding, genetics and genomics. The present review mainly focuses on the production of doubled haploids in rice through *in vitro* anther culture, factors affecting rice androgenesis, its

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advantages and applications in plant breeding and genetics.

In-vitro androgenesis in rice

Rice (*Oryza sativa* L.) is the carbohydrate source for more than half of the world's population. In the last few decades, there has been tremendous increase in rice productivity with the development of high yielding varieties (Mohiuddin et al, 2014). Recently, hybrid rice technology has come up as a viable option to manifold the rice yield. However, despite its yield advantage over inbreds, the hybrid rice technology has not found favour with the farmers of India and other developing nations due to high cost and poor grain quality. The isolation of doubled haploid indica hybrid lines through *in vitro* anther culture with high yielding and superior grain quality has been successful to some extent in solving the problems associated with hybrid rice breeding (Mishra et al, 2013).

In rice, haploids were first produced through anther culture by Niizeki and Oono (1968). Rice is a unique material in which around 30%–40% of the regenerant from the anther culture are doubled haploids due to the spontaneous doubling of the haploids (Mishra et al, 2013). Rice anther culture is a two-step process of initial development of calli and subsequent regeneration of green plants from embryogenic calli (Fig. 1). Rice panicles are collected during the booting period, when the microspores are at mid- to late-uninucleate stages. Previous studies have shown that florets having anther length of less than half of the size contain anthers having microspores of mid- to late-uninucleate stage (Niroula and Bimb, 2009). The boots (young panicles still enclosed within the flag leaf sheath) are wiped with a clean muslin cloth moistened with 70% alcohol. The wrapped boots are then cold pre-treated at 10 ºC for 8–10 d. The spikelets are surface sterilized using 20% commercial bleach (containing 4% NaClO) for 5 min and rinsed three times with sterile de-ionized water. Cytological examination of microspore stage in the anthers is conducted and 20–25 anthers with microspores at mid-uninucleate to early bi-nucleate stages are uniformly dusted over the surface of the media. The inoculated anthers are incubated in dark at (25 ± 1) ºC and observations on the anther response to callus induction were recorded starting from 3–4 weeks after inoculation. Then, the calli are transferred onto the regeneration medium and incubated under artificial light (about 2 000 lux) at (25 ± 1) ºC for callus regeneration. The green plantlets are transferred to rooting medium for root formation. The plants with well-formed roots are transferred to pots in a greenhouse (Mishra et al, 2013).

Cytogenetic characterization has revealed that the anther derived plants have different ploidy levels (Sah and Niroula, 2007). Counting of chromosome numbers directly by cytological observation is the most accurate way for identification of ploidy level. However, recently, the ploidy level in plants is estimated by measuring the C-value (amount of DNA in the unreplicated gametic nucleus using flow cytometry) (Ochatt et al, 2009). Ploidy is also confirmed by pollen grain size. High ploidy levels are

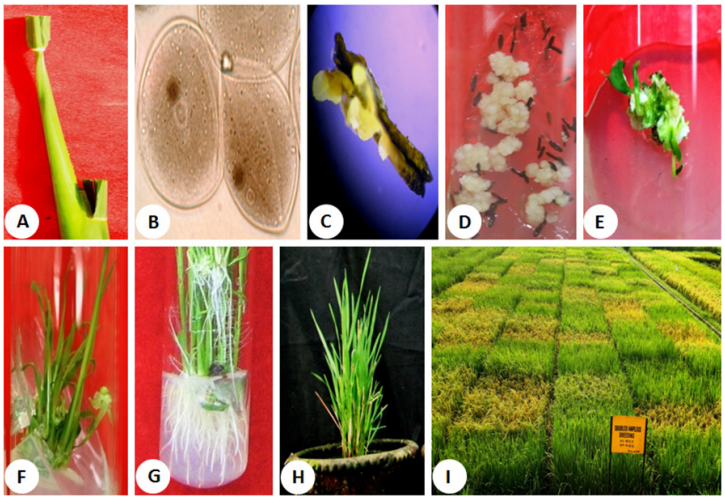

Fig. 1. *In vitro* androgenesis in rice.

A, Boot leaf collected having microspores at mid- to late-uninucleate stage; B, Cytological confirmation of mid- to late-uninucleate stage; C, Microscopic view of anther with multiple calli; D, Callus induction; E, Callus regeneration; F, Green plant regeneration; G, Rooting; H, Anther derived plants in net-house condition; I, Anther derived plants in field for agronomic evaluation.

associated with large pollen grains, while the number of pollen grains is not different between ploidy levels (Fukuhara, 2000).

Factors affecting rice androgenesis

Although anther culture is recognized as a valuable tool in plant breeding programs, various factors limit its actual application, including high genotypic dependency, low frequencies of callus induction and plant regeneration, and high frequency of haploid plants (Medhabati et al, 2014). Indica rice varieties have a limited response to anther culture because of early anther necrosis, poor callus proliferation and regeneration of albino plantlets (Chen et al, 1991). Significant varietal differences were reported in rice anther culture, and the anther culture ability of japonica varieties is generally higher than that of indica ones (He et al, 2006). Numerous endogenous and exogenous factors that affect the success of anther culture in rice are as follows.

Genotype

Among the endogenous factors reported so far, genotype is a major one that affects *in vitro* androgenic response. Lentini et al (1995) reported that only 1 out of 35 indica varieties exhibits pollen callusing on N_6 medium. Poor callusing and regeneration abilities, and high percentage of albino plants have affected the androgenic response of indica rice, limiting its utilization as a breeding tool (Silva, 2010). Genotypic variations in callus induction and subsequent plant regeneration potential in rice have been extensive studied by many researchers (He et al, 2006; Bagheri and Jelodar, 2008). However, recent studies have highlighted the genotype specificity of anther culture response within indica subspecies, by using improved culture media (Ratheika and Silva, 2007; Talebi et al, 2007). Niroula and Bimb (2009) reported the possibility of enhancing the anther culturability by manipulating the medium compositions in some responsive Nepalese rice varieties. Further, high doses of nitrogen, phosphorus and potassium can be imperative at both stages of androgenesis in indica rice (Silva, 2010). Additionally, the interactions of genotypes, culture media, and culture temperatures also play important roles in the activation of recalcitrant genes for callus induction and regeneration (Javed et al, 2012). These studies suggest that selection of better responsive rice genotypes and manipulation of the non-genetic factors like culture medium components and pre- and post-culture conditions can enhance the anther cultarability in rice.

Physiology of donor plants

Growth conditions and development of donor plants determine the physiological characteristics of anthers in the panicle (Szarejko, 2003). Varying temperatures during the booting stage of donor plants are shown to affect the normal development of microspores *in vivo* as well as the androgenesis of microspores *in vitro* (Raina, 1989). Besides temperature, sufficient nitrogen supply is also required to maintain the photosynthetic activity of the plants and thus ensuring pollen development. Optimum level of nitrogen in donor plants results in anthers having well superior microspores with high embryogenic ability (Lapitan and Violeta, 1999). Veeraraghavan (2007) observed that the plants grown in the fields are significantly superior to those grown in the glasshouse or pots. The maturity of the donor plant also plays a vital role in affecting the anther culture response. Anthers from the top part have the lowest callus formation frequency compared to the middle and bottom parts (Jacquard et al, 2006). Hence, the androgenic response can be enhanced by carefully nurturing the donor plants and growing them under favourable environmental conditions.

Developmental stage of anther

The pollen staging can contribute to the enhancement of anther culture efficiency by correctly identifying the maturity stage of anthers in indica rice varieties (Silva and Ratnayake, 2009). In rice, anthers with the early or mid- to late-uninucleate pollen stages of microspore development are the most responsive and suitable for culture (Datta and Wenzel, 1998). The difficulty in culturing the older stages of pollen may be due to their commitment to differentiation into a male gametophyte. The response of anthers at the tetrad stage is not good at all, and it falls sharply after the first pollen mitosis. At this stage, starch deposition begins, but no sporophytic development and subsequently no macroscopic structures form in the microspores. However, pollen at this stage, when cold pre-treated, can deviate from normal developmental programme and switch to sporophytic growth. Therefore, determining the developmental stage of pollen in the anthers is important to optimize the anther culture response of a given rice genotype (Cha-um et al, 2009; Silva, 2010). Bishnoi et al (2000) reported that the distance from flag leaf to penultimate leaf auricle can

be a convenient morphological marker for estimating the maturity stage of pollen in different rice varieties.

Pre-treatment

Stress application during the developmental period of pollen grains, such as temperature pre-treatment, osmotic shock and sugar starvation, is highly essential for the induction of androgenesis. However, the type, duration and time of these pre-treatments may vary with the species or even variety (Datta, 2001). Cold pre-treatment is reported to enhance the stoppage of the gametophytic development of microspores during cold shock stress and guide the continuous division of the microspores into forming callus/embryo i.e. a sporophyte during culture (Touraev et al, 1996). This shift from gametophytic to sporophytic mode of development may cause instability and the loss of chlorophyll is a manifestation of that shift. The positive effects of cold pre-treatment on callus induction include delay of anther wall senescence, increase of symmetric division of pollen grains and release of substances necessary for androgenesis, mainly amino acids and shock-thermic proteins (Kiviharju and Pehu, 1998). Several researchers reported a stimulatory effect of low temperature shock on the androgenic response in several species (Silva and Ratnayke, 2009; Gueye and Nidr, 2010; Sen et al, 2011). Cold pre-treatment at 8 ºC for 14 d is the most effective for anther culture in selected indica, japonica rice varieties and inter sub-specific hybrids (Herath et al, 2009), and a pre-treatment at 10 ºC for 7–9 d has a positive influence on elite indica rice hybrids (Mishra et al, 2013). Recently, it was reported that cold pre-treatment at 12 ºC for 5 d give the best performance for callus induction and plant regeneration in 13 indica rice genotypes (Kaushal et al, 2014). In contrast, exposure of rice calli to high temperatures at meiosis easily forms albino plants, suggesting that optimal temperature for regeneration appearing to be genotype dependent (Ouyang et al, 1983). High temperature pre-treatment disrupts the normal integrated development of somatic anther tissue and subsequently synchronizes the physiological states of the two tissues, thereby stimulating the induction process (Dunwell et al, 1983). Heat pre-treatment has been used to induce embryogenesis from isolated anthers, as it disrupts the cytoskeleton in microspores at the initial phase (Ferrie and Keller, 1995).

Osmotic stress

Osmotic stress or loss of cellular water content often disrupts the plasmodesmatal connections between the pre-embryonic cells, making the cells physiologically isolated and allowing a greater number of cells to differentiate (Wetherell, 1984). Several reports have shown that mannitol induced osmotic stress in microspore culture enhances the green plant regeneration frequency in indica and japonica varieties (Cistue et al, 1994; Raina and Irfan, 1998). Ogawa et al (1995) reported that sugar starvation of the anthers of IR43 for 2 d at the beginning of culture causes promotion of androgenesis (39%, 12-fold), which is more than the other mannitol pre-treatments. Agarose concentration increased from 0.5% to 1.0% produces over 8-fold increase in shoot regeneration in indica rice varieties IR43 and Pusa Basmati 1 (Silva and Ratnayake, 2009).

Gamma radiation

Gamma radiation given to anthers prior to culture has proved beneficial (Zapata and Aldemita, 1989). Mkuya et al (2005) reported prolific doubled haploid production and green plant regeneration in indica rice line TM7-5 upon irradiation with ^{137}Cs gamma rays at 20 Gy. Earlier, it was demonstrated that the gamma ray dose of 20 Gy has significant stimulation effect on regeneration of green plants from rice anther culture (Chen et al, 2001).

Culture medium

Culture medium is an important aspect in androgenesis of cereals as it provides nutrition and also decides the fate of microspores. N_6 (Chu, 1978) and MS (Murashige and Skoog, 1962) are the two most widely used media for anther culture of cereals. However, by 1978, several medium formulations are reported inducing N_6-like Heh-2, Heh-5, SK-3, SK-8, Szechuan medium, Medium V and Chaleffs R-2. Low inorganic ammonium nitrogen (NH_4^+) had generally proven to be better for androgenesis in cereals and based on this, N_6 medium containing low NH_4^+ was designed and has been most widely used for rice anther culture (Chu et al, 1975). However, indica varieties require even lower amount of NH_4^+. Raina and Zapata (1997) studied the nitrogen requirement of indica rice variety IR43 and developed the MO19 medium suitable for androgenesis of indica rice. N_6 media supplemented with organic adjuvants like yeast extracts, casein hydrolysate and coconut water show enhanced androgenic callus induction in indica rice varieties (Roy and Mandal, 2005).

Sucrose has been used as a major carbohydrate source in the induction medium. Sucrose level of 2%–5% has been suggested to be appropriate for rice anther culture (Reinert and Bajaj, 1977). Sucrose concentration above 6% in the induction medium often increases the proportion of albino plants (Wang et al, 1978). However, the substitution of sucrose with maltose substantially increases the formation of embryo-like structures in all genotypes (Navarro-Alvarez et al, 1994). The toxicity of sucrose for androgenesis is due to the sensitivity of microspores to fructose but not to glucose. Maltose has been reported to be a superior source of carbohydrate than sucrose for androgenesis in several species, including cereals (Sen et al, 2011). Correlation analysis between the maltose concentration in media and the frequency of albino plants revealed that the application of the maltose minimizes the frequency of albino plants from anthers (Park et al, 2013). Sorbitol has also been reported to enhance the plant regeneration of rice when applied to the regeneration medium (Yoshida et al, 1994). Besides, amino acids have also been used as nitrogen source in *in vitro* culture of various tissues for enhanced green plant regeneration and production (Ogawa et al, 1995). According to Zhao et al (2002), $AgNO_3$ is known to interfere with ethylene action and form complexes which inhibit ethylene responses in anther culture program. Faruq et al (2014) also reported that the addition of $AgNO_3$ to the medium improves callus induction and plant regeneration in indica rice.

Growth regulator

The growth regulators, mainly auxins and cytokinins, are known to control the dedifferentiation processes in *in vitro* cultures of crop plants. The rate of success can be enhanced by improving the composition of tissue culture medium by manipulating the plant growth regulators (Mandal and Gupta, 1995). Among the auxins, 2,4-dophenoxy acetic acid (2,4-D) and naphthalene acetic acid (NAA) are the most commonly used growth regulators for induction of callus from rice anthers (Trejo-Tapia et al, 2002). Indole acetic acid (IAA) and NAA may induce direct androgenesis, while 2,4-D promotes rapid cell proliferation and formation of non-embryogenic callus (Ball et al, 1993). Moreover, 2,4-D results in high callus induction and the 2,4-D induced calli produce higher green plant than the NAA induced calli. Moreover, 2,4-D inhibits the organogenesis of calli, and NAA promotes the formation of roots and sometimes completes plants (Cornejo-Martin and Primo-Millo, 1981). However, neither 2,4-D nor NAA can support regeneration, and the use of cytokinins like kinetin and benzyl amino purine are required (Mandal and Gupta, 1995).

Gelling agent

Agar is the most common gelling agent used to solidify medium, being cheap and easily available, but the negative effect of agar is the release of impurities from agar into the medium, hampering the growth and development. To avoid this problem, agar can be replaced by alternative gelling agents. Ficoll is first recommended as a buoyancy agent to prevent embryoids from sinking in anther culture (Kao, 1981). Ficoll is a non-ionic, synthetic polymer of sucrose which increases the surface density. Ficoll supplement improves the ratio of green to albino plantlets as confirmed by Lashermes (1992). However, ficoll also changes medium osmotic potential. Lee and Lee (1995) studied the effect of gelling agents on rice anther culture and found gel rite to be the most effective one.

Application of doubled haploids in rice breeding, genetics and genomics

Doubled haploid lines are homozygous, immortal and true breeding lines, which are suitable for breeding purposes. This system provides an unparalleled opportunity to shorten the breeding cycle and fix agronomic traits in the homozygous state, such as recessive genes for disease resistance (Datta, 2005). Plant breeders need to adapt speed and efficiency to develop new varieties, which is important for the breeding industry (Tuvesson et al, 2007). The importance of doubled haploids has been recognized by plant breeders in plant biology and genetics (Suriyan et al, 2009), genome mapping (Hussain et al, 2012) or genetic manipulation, as part of genetic transformation or quantitative trait loci (QTLs) analysis (Chauhan and Khurana, 2011). Previous reports have shown that DH rice lines are more viable and more than 100 rice breeding lines or varieties have been developed through anther culture in China and several antherdevived lines have been reported in India, Japan, South Korea, Hungary and USA (Siddique, 2015). Several rice DH varieties with superior grain quality characteristics, resistant to diseases like blast, bacterial blight, brown planthopper and tolerance to abiotic stress have been released in different countries (Table 1).

The utility of DH breeding in indica rice in India was firstly demonstrated with the release of two varieties, Satyakrishna (CR Dhan 10) and Phalguni (CR Dhan 801) (CRRI Annual Report, 2008, 2010). CR Dhan 10 has a duration of 135 d with erect, non-lodging, semi-dwarf (105 cm) plant type and yields of 5.0 t/hm^2 in wet season and 6.0 t/hm^2 in dry season. CR Dhan 801 has a duration of 115–120 d with erect, non-lodging, semi-dwarf plant type with the productivity levels ranging from 4.0–4.5 t/hm^2 in wet season and 5.0–6.0 t/hm^2 in dry season. CR Dhan 10 has been recommended for cultivation in irrigated- and rain-fed shallow low land ecologies, while CR Dhan 801 is suitable for cultivation in irrigated as well as bunded upland ecologies.

The first salt tolerant rice variety IR51500AC11-1 as PSBRc50 'Bicol' derived through anther culture was recommended for commercial cultivation in salt-affected rice lands (Senadhira et al, 2002). Lee et al (2003) developed a salt-tolerant DH rice variety through anther culture of six F_1 hybrids obtained by back-cross or three-way cross between indica and japonica varieties differing in salt tolerance. Similarly, Thomson et al (2010) developed DH lines from the crosses involving salt-tolerant rice lines from International Rice Research Institute (IRRI) and identified a DH line AC-1 suitable for cultivation in saline areas of Bangladesh. Rice anther culture can be used to produce DHs with multiple stress tolerances. Dewi et al (2009) reported the anther culturarability of indica genotypes used for development of new rice varieties tolerant to aluminium toxicity. Similarly, Purwoko et al (2010) reported the use of anther culture in upland rice breeding program by producing DH lines tolerant to aluminium stress, shade and blast resistance.

DH lines are ideal for genetic mapping because of their homozygosity and uniformity, and each line can be replicated indefinitely over several locations (Tinker et al, 1996). DH populations have played a major role in facilitating the generation of molecular marker maps in eight crop species (Maluszynska, 2003). In rice, linkage map developed using a DH population has led to the identification of molecular markers linked with major genes for resistance to rice blast, bacterial blight, and sheath blight disease (Wang et al, 2001). The integration of the genetic and physical maps on DH population provided the necessary precision in targeting candidate resistance genes against the diseases (Wang et al, 2001). Similarly, chromosome maps have been established in a range of species including rice, rapeseed and wheat by using DHs (Forster and Thomas, 2005).

A DH system plays a vital role in inducing and fixing mutations (Szarejo and Forster, 2007). It enhances the mutation induction and selection efficiency (Szarejko, 2003) and is valuable to fix and express desirable recessive traits introduced through mutation or hybridization, thereby enriching the germplasm

Table 1. List of released doubled haploid rice varieties generated through anther culture.

Variety	Characteristic	Country	Reference
Huayu I, Huayu II, Xin Xiu, Late Keng 959, Tunghua 1, Tunghua 2, Tunghua 3, Zhonghua 8, Zhonghua 9, Huahanzao, Huajian 7902, Tanghuo 2, Shanhua 7706, Huahanzao 77001, Nanhua 5, Noll, Hua 03	High yielding varieties with superior grain quality; resistant to blast and bacterial blight diseases	China	Zang, 1980; Hu and Zeng, 1984; Chen, 1986; Loo and Xu, 1986; Yang and Fu, 1989
Guan 18	Early maturity; good quality and disease resistance	China	Zhu and Pan, 1990
Huayu 15	Resistant to lodging and diseases; good quality	China	Shouyi and Shouyin, 1991
Milyang 90	Good grain quality; resistant to brown planthopper and stripe virus disease	China	Chung, 1987
Hwacheongbyeo, Joryeongbyeo, Hwajinbyeo	Resistant to brown planthopper, rice stripe tenuivirus, blast and bacterial blight	South Korea	Lee et al, 1989
Bicoll (IR51500AC11-1)	Salt tolerant	the Philippines	Senadhira et al, 2002
Parag-401	Superior grain quality and resistant to iron chorosis	India	Patil et al, 1997
Risabell	High milling and cooking quality; resistant to blast	India	Pauk et al, 2009
Janka	Drought tolerance; good grain quality	India	Pauk et al, 2009
Abel	Cold tolerance at early stage	India	Pauk et al, 2009
CR Dhan 10 (CRAC2221-43), Satyakrishna	Resistant to neck blast, sheath-rot and yellow stem borer	India	CRRI Annual Report, 2007–2008
CR Dhan 801 (CRAC2224-1041, IET 18720), Phalguni	Resistant to leaf blast, gall midge; moderately resistant to sheath rot, rice stripe tenuivirus, yellow stem borer, brown spot and sheath blight	India	CRRI Annual Report, 2009–2010

(Jiang et al, 2002). High frequency of rice mutants has been generated in rice varieties treated with ethyl methane suphonate (EMS) at 10 and 20 d after anther inoculation (Lee and Lee, 2002). Gamma irradiated mutagenic lines when subjected to anther culture resulted in stable mutants with significantly higher and earlier flowering than the parent (Myint et al, 2005).

Quantitative trait loci (QTLs) govern major agronomic traits in cultivated rice (Datta, 2005). As the effects of QTLs are small and highly influenced by the environmental factors, accurate phenotyping with replicated trials are needed. This is possible by doubled haploids because of their true breeding nature and convenience of producing large numbers. Six QTLs associated with resistance to brown planthopper in rice were mapped by using a DH mapping population derived from the cross between IR64 and Azucena (Soundararajan et al, 2004). Similarly, QTLs linked to sheath blight resistance were detected by using DH populations of Maybelle, an American japonica variety, and Baiyeqiu, a Chinese indica landrace (Xu et al, 2011). QTL mapping of root traits was done by using a DH population derived from a cross between upland and lowland japonica rice in three environments (Li et al, 2003). DH population derived from a cross between japonica rice Chunjiang 06 and indica rice TN1 was used for identification of five QTLs for panicle-layer-uniformity (Ma et al, 2009a). Earlier, Hittalmani et al (2002) reported the mapping of QTLs for plant growth, yield and yield-related traits across three diverse locations in a DH rice population. Recently, QTL analysis of yield components in rice was performed and two QTLs affecting yield and yield components were identified by using a DH population of Cheongcheong (indica) and Nagdong (japonica) (Park et al, 2014). The same DH population was also used to map four QTLs related to amylose content, two QTLs related to protein content and two QTLs associated with lipid content for rice quality analysis (Lee et al, 2014).

Application of DHs in cross-pollinated species improves the selection efficiency by overcoming problems of inbreeding depression and production of homozygous lines during conventional breeding through rapid fixation of genes in one generation and early elimination of deleterious alleles from populations (Murovec and Bohanec, 2012). In backcross breeding, identifying the lines carrying the target trait at each generation is a major problem. The problem is particularly acute if the target trait is recessive, due to selection among the heterozygotes. The combination of doubled haploid and molecular marker is highly effective in solving this problem. Bulked segregant assay is dependent on accurate phenotyping and the DH population has particular advantage because they are homozygous or pure lines and can be tested repeatedly. DH populations are commonly used in bulked segregant analysis, which is a popular method in marker assisted breeding (William et al, 2002).

Grain quality has always been an important consideration in rice variety selection and development (Babu et al, 2013). The major constraints that limit the large scale adoption of hybrid rice cultivars are the high seed production cost and the poor grain quality of the hybrids (Janaiah and Hossain, 2003). Anther culture technique offers great opportunity for accelerating breeding progress and improving grain quality characters (Xa and Lang, 2011). DH lines derived from rice variety Koshihikari show superior visual grain quality and excellent eating quality (Xa and Lang, 2011). Milyang 90, an anther culture derived line with superior grain quality and overall good performance, was isolated from 2 000 breeding lines (Chung, 1987). Guan 18, an indica rice hybrid with superior grain quality characteristics, is a great achievement of anther culture quality breeding (Zhu and Pan, 1990).

Plant breeders are also aiming at grain's nutritional quality improvement. Iron deficiency alone affects more than three billion people in the world. Zinc deficiency is commonly associated with diarrhoea, pneumonia, and even causes death. These can be overcome by increasing the grain micronutrient content of existing and future high yielding indica varieties using high zinc, high-iron japonica donors, which are known to be highly responsive to anther culture. IRRI has embarked on an ambitious project supported by the Harvest Plus Challenge Program to produce nutritious rice. Generation of DH lines is an integral part of this mission. More than 1 500 DH lines through anther culture have been evaluated for their agronomic potential and for high iron and zinc contents (Grewal, 2009).

The purity of parental lines used in developing hybrid rice strongly decreased all these years because of gene drift, mutation, artificial and biological confounding, and exposure to unfavourable natural stress. Such a decrease has resulted in reduced yield

potential and grain quality. The conventional technique of purification is elaborate and time consuming, and selection is made on phenotype which cannot guarantee that selected materials are genetically homozygous and stable. Because anther culture can make genes highly homozygotic, it is a more effective method for purification (Zhu et al, 1998). Bai et al (1991) reported that hybrids derived from anther culture purified restorer line Minghui 63 is significantly improved in purity, seed-setting rate, yield potential and resistance. Similarly, Wang et al (1994) adopted anther culture procedure to create new restorer lines for cytoplasmic male sterility.

Major drawback of rice androgenesis

A serious drawback of androgenesis, either in anther or microspore culture, is the incidence of albinism where most or all regenerated plants have been found to be useless albino (Grewal et al, 2009; Khatun et al, 2012). In general, albino plants contain deleted forms of the plastid genome and they are devoid of 23S and 16S rRNA (Zubko and Day, 2002). The basic cause of albinism in rice is impairment of DNA in plastids or nuclei or in both of them (Kumari et al, 2009). Several factors, including pre-treatment, culture medium and the stage of the pollen, affect the frequency of albinos and the frequency varies from 5% to 100% (Talebi et al, 2007). Albino plant generation is specific to anther culture because only a few albino plants are generated from somatic cells (Tsukahara et al, 1996). QTLs on chromosomes 9 and 10 have been identified which control the frequency of albino plants among regenerated rice plants (Yamagishi et al, 1998). Albinism can be considerably reduced by shortening the culture period (Asaduzzaman et al, 2003). Touraev et al (2009) have reported the increased microspore survival with reduction in frequency of albino production when starvation and cold stress being applied simultaneously for shorter periods (3–4 d). Notwithstanding the fact that albinism decreases the efficiency of doubled haploid, and a systematic anther culture approach with the use of suitable media and nutrients can lead to sufficient DH production for specific traits.

CONCLUSIONS AND PROSPECTS

The development of *in vitro* techniques for production of haploids was a major feat in the field of biotechnology and plant breeding in the past few decades. It is concluded that DH technology plays an important role in the field of plant breeding, genetics and genetic engineering. The ability to shorten the breeding cycles and production of complete homozygous plants makes the technology best for cultivar development, back crossing, genome mapping, QTL mapping, quantitative genetics, genomics, gene identification, gene discovery and transgenic plant development. In addition to plant breeding programs, it also provides great opportunities in improving the grain quality and its nutritional value to overcome the malnutrition problem. The homozygous lines are of utmost importance in hybrid rice breeding. The genetic make-up of indica rice is a deciding factor in achieving success in anther culture response. So, efforts should be made to understand the genetic mechanism that controls the anther culturability, particularly in the recalcitrant indica rice through genetic engineering and molecular genetic studies. It is also very important to have a better understanding of the fundamental processes involved in microspore embryogenesis and identifying the important genes involved in reprogramming the microspores leading to embryogenesis. Furthermore, the haploid induction technique can nowadays be efficiently combined with several other plant biotechnological techniques, enabling several novel breeding achievements, such as hybrid breeding, improved mutation breeding, reverse breeding, and genetic transformation.

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